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CITATION:

Matsui, Masafumi ...[et al]. Unexpected Phylogenetic Position of Parapelophryne among Southeast Asian Bufonids as Revealed by Mitochondrial DNA Sequence (Amphibia, Anura, Bufonidae). *Current Herpetology* 2015, 34(2): 182-187

ISSUE DATE:

2015-08

URL:

<http://hdl.handle.net/2433/216861>

RIGHT:

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## Unexpected phylogenetic position of *Parapelophryne* among Southeast Asian bufonids as revealed by mitochondrial DNA sequence (Amphibia, Anura, Bufonidae)

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**Abstract:** We estimated the phylogenetic relationships of an enigmatic small toad *Parapelophryne scalpta* from Hainan Island, China to nine other bufonid genera from Southeast and East Asia using ca. 2000 bp sequences of the mitochondrial DNA genes 12S rRNA, tRNA<sup>val</sup>, and 16S rRNA using maximum likelihood and Bayesian inference methods. The East and Southeast Asian bufonid genera formed a clade in which seven lineages with unresolved relationships to each other were recognized. Monophyly was supported only for (A) *Parapelophryne* and *Bufo*, (B) *Phrynoidis* and *Pedostibes*, and (C) *Leptophryne* and *Ansonia*. All genera were genetically divergent from each other and *Parapelophryne*, erected purely based on morphology, could be recognized as a distinct genus. On the other hand, it was found to be the sister genus of East Asian *Bufo*, an unexpected result given their great morphological difference and discontinuous distribution.

Key words: *Bufo*; China; *Parapelophryne*; *Pelophryne*; Phylogeny

### INTRODUCTION

Bufonidae Gray, 1825 is a large, nearly cosmopolitan toad family consisting of 580 species (Frost, 2015). Southeast Asian members of the family were long considered to include only five genera (*Bufo* Garsault, 1764; *Ansonia* Stoliczka, 1870; *Leptophryne* Fitzinger, 1843; *Pedostibes* Günther, 1876; and

*Pelophryne* Barbour, 1938; Inger, 1966; Frost, 1985), but recent studies, mainly based on molecular phylogeny, split Southeast Asian *Bufo* into three genera (*Duttaphrynus* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green, and Wheeler, 2006; *Ingerophrynus* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green, and Wheeler, 2006; and *Phrynoidis* Fitzinger in Treitschke, 1842:

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Frost et al., 2006). Phylogenetic relationships of these genera with other members from outside the region have been well studied (Frost et al., 2006; Pyron and Wiens, 2011).

One exception is the position of *Parapelophryne* Fei, Ye, and Jiang, 2003, a monotypic genus composed of *P. scalpta* (Liu and Hu, in Liu, Hu, Fei, and Huang, 1973), endemic to Hainan Island of southern China (Fei et al., 2005, 2012). The species was originally described by Liu et al. (1973) as a member of *Nectophryne* Buchholz and Peters, 1875, then moved to *Pelophryne* (Ye and Fei, 1978), and finally accommodated in a distinct genus *Parapelophryne* (Fei et al., 2003).

It is interesting and important to elucidate the phylogenetic position of *Parapelophryne* among allied bufonid genera from Southeast Asia to understand their evolutionary history in this region. We therefore analyzed mitochondrial gene sequences of *P. scalpta* and estimate the relationships by comparing the data with sequences of the allied genera that have already been clarified (Matsui et al., 2010, 2012).

## MATERIALS AND METHODS

We examined DNA sequences of 12S rRNA, tRNA<sup>val</sup>, and 16S rRNA genes from

two specimens of *P. scalpta* collected from Hainan Island during surveys conducted by the Kadoorie Farm and Botanic Garden Team and stored in 95% ethanol. The vouchers are temporarily kept at the Kyoto University, Graduate School of Human and Environmental Studies (KUHE). We compared the sequences thus obtained with representative species of eight other Southeast Asian bufonid genera (*Ansonia penangensis* Stoliczka, 1870; *Duttaphrynus melanostictus* [Schneider, 1799]; *Ingerophrynus parvus* [Boulenger, 1887]; *Leptophryne borbonica* [Tschudi, 1838]; *Pedostibes hosii* [Boulenger, 1892]; *Pelophryne signata* [Boulenger, 1895]; *Phrynoidis asper* [Gravenhorst, 1829]; *Sabahphrynus maculatus* [Mocquard, 1890]), one East Asian species (*Bufo japonicus formosus* Boulenger, 1883), and two distinctly distant (Frost et al., 2006) outgroup species (a bufonid, *Atelopus flavescens* Duméril and Bibron, 1841 and a dendrobatid *Dendrobates auratus* [Girard, 1855]; Table 1).

Methods for phylogenetic analyses followed Matsui et al. (2010). The PCR cycling, purification, and sequencing procedures were identical to those described by Matsui et al. (2010). The resultant new sequences were deposited in GenBank (LC061221–061223; Table 1). The alignment matrix with 2066

TABLE 1. Sample of East and Southeast Asian bufonid species used for DNA analysis in this study together with the information on voucher, collection locality, and GenBank accession numbers. Voucher abbreviations: BORN=BORNEENSIS Collection, University Malaysia Sabah; KUHE=Graduate School of Human and Environmental Studies, Kyoto University.

Species	Voucher	Locality	GenBank	Reference
<i>Parapelophryne scalpta</i>	KUHE 49653	Hainan, China	LC061221	This study
<i>Parapelophryne scalpta</i>	KUHE 49654	Hainan, China	LC061222	This study
<i>Pelophryne signata</i>	KUHE 53200	Kubah, Sarawak	AB746456	Matsui et al. (2012)
<i>Ansonia penangensis</i>	KUHE UNL	Penang, Malaysia	AB435262	Matsui et al. (2010)
<i>Sabahphrynus maculatus</i>	BORN 08425	Crocker, Sabah	AB331718	Matsui et al. (2007)
<i>Pedostibes hosii</i>	BORN 22088	Tawau, Sabah	AB331717	Matsui et al. (2007)
<i>Leptophryne borbonica</i>	KUHE 53887	Penrissen, Sarawak	AB746458	Matsui et al. (2012)
<i>Ingerophrynus parvus</i>	KUHE 39047	Penang, Malaysia	AB746455	Matsui et al. (2012)
<i>Duttaphrynus melanostictus</i>	KUHE 10524	Marudi, Sarawak	AB331714	Matsui et al. (2007)
<i>Phrynoidis asper</i>	KUHE 39025	Penang, Malaysia	AB746454	Matsui et al. (2012)
<i>Bufo japonicus formosus</i>	KUHE 45512	Shiga, Japan	LC061223	This study
<i>Atelopus flavescens</i>	—	French Guiana	DQ283259	Frost et al. (2006)
<i>Dendrobates auratus</i>	—	—	AY326030	Darst and Cannatella (2004)

nucleotide sites was subjected to estimation of phylogenetic relationships using maximum likelihood (ML) and Bayesian inference (BI). In the BI analysis, two independent runs of four Markov chains were conducted for ten million generations, and the first three million generations were discarded as burn-in. Pairwise comparisons of uncorrected sequence divergences (p-distances) in 16S rRNA were also calculated. Details of these procedures are given in Matsui et al. (2010).

RESULTS

Of 2066 nucleotides generated, 717 were variable, and 394 were parsimony-informative. The best substitution model for ML derived from Kakusan4 (Tanabe, 2011) was the J2 model (Jobb, 2011) with a gamma shape parameter (estimated gamma values were 0.240 for 12S rRNA, 0.471 for tRNA<sup>val</sup>, and 0.265 for 16S rRNA), and the likelihood value of lnL -13025.376, while for Bayesian analysis it was the general time reversible model (GTR:

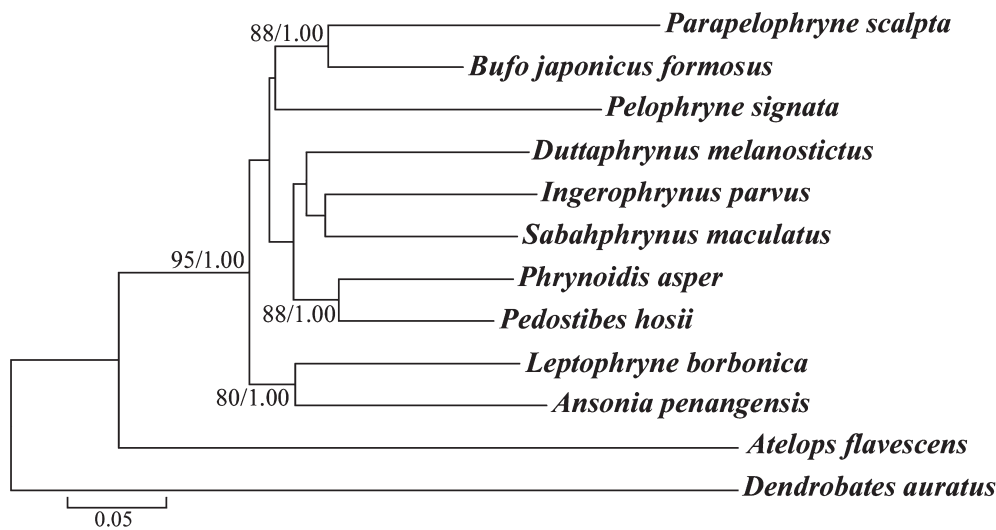


FIG. 1. ML tree from a 2066 bp sequence of mitochondrial 12S rRNA, tRNA<sup>val</sup> and 16S rRNA genes for *Parapelophryne scalpta* and samples of East and Southeast Asian bufonid species studied. Numbers above or below branches represent bootstrap supports for ML inferences and Bayesian posterior probabilities (ML-BS/BPP). Only ML-BS>70% and BPP>0.95 are shown.

TABLE 2. Uncorrected p-distances (%) in ca. 1200 bp of 16S rRNA among *Parapelophryne scalpta* and samples of East and Southeast Asian bufonid species studied.

	1	2	3	4	5	6	7	8	9	10	11
1 <i>Parapelophryne scalpta</i> KUHE 49653											
2 <i>Bufo japonicus formosus</i> KUHE 45512	13.3										
3 <i>Pelophryne signata</i> KUHE 53200	14.9	12.9									
4 <i>Ansonia penangensis</i> KUHE UNL	16.7	12.7	12.2								
5 <i>Leptophryne borbonica</i> KUHE 53887	14.9	13.2	12.9	12.3							
6 <i>Sabahphrynus maculatus</i> BORN 08425	15.5	11.8	12.9	12.7	13.1						
7 <i>Pedostibes hosii</i> BORN 22088	15.0	11.5	12.4	11.8	11.7	10.5					
8 <i>Phrynoidis asper</i> KUHE 39025	15.4	11.5	13.9	12.7	14.0	11.7	10.0				
9 <i>Ingerophrynus parvus</i> KUHE 39047	14.3	12.1	12.3	12.1	13.2	10.9	11.5	12.0			
10 <i>Duttaphrynus melanostictus</i> KUHE 10524	14.9	11.3	12.5	12.4	12.7	11.3	11.3	12.0	12.1		
11 <i>Atelopus flavescens</i> DQ283259	20.7	17.6	19.3	17.2	17.1	17.8	17.0	18.0	18.0	17.0	
12 <i>Dendrobates auratus</i> AY326030	23.4	21.4	23.1	20.8	22.4	20.9	20.8	20.4	20.9	20.8	20.4

Tavaré, 1986) with a gamma shape parameter (estimated values of 0.267, 0.636, and 0.286, respectively), and the likelihood value of  $\ln L$  -13049.567.

Phylogenetic analyses employing two different optimality criteria yielded similar topologies with respect to significantly supported branches, and only the ML tree is presented in Fig. 1. Monophyly of Asian bufonid genera with respect to *Atelopus* and *Dendrobates* was supported (ML BS=95%, BPP=1.00). Relationships among bufonid genera are generally unresolved and seven lineages were recognized. Of these, each of *Parapelophryne* and *Bufo* (ML BS=88%, BPP=1.00), *Phrynoidis* and *Pedostibes* (ML BS=88%, BPP=1.00), and *Leptophryne* and *Ansonia* (ML BS=80%, BPP=1.00) formed a clade. From the other species employed, *Parapelophryne* exhibited substantially large uncorrected p-distances in 16S rRNA, ranging from 13.3% (between *Bufo*) to 16.7% (between *Ansonia*). This minimum value was exceeded only by those between *Phrynoidis* and *Pelophryne* (13.9%) or *Phrynoidis* and *Leptophryne* (14.0%) (Table 2), and indicated distinct generic status of *Parapelophryne* among East and Southeast Asian bufonids.

## DISCUSSION

*Parapelophryne scalpta* was originally described by Liu and Hu (1973) as a member of *Nectophryne*, because the authors considered the species to be allied to some small Southeast Asian bufonids, and after consulting old literature (Boulenger, 1882; Noble, 1931), the authors treated it as *Nectophryne*, which is confined to Central to West Africa (Frost, 2015), far from Hainan.

Because Barbour (1938) already split Southeast Asian *Pelophryne* from African *Nectophryne* and the subsequent authors followed this idea (Inger, 1954, 1960), Ye and Fei (1978) moved the species to *Pelophryne* based on general similarity between them, while noting presence of important differences including the number of presacral vertebrae

(Fei, 1999).

The genus *Pelophryne* occurs in the Malay Peninsula, Borneo, and the Philippines (Frost, 2015), and no species has been found in the Indochina region, which is intervening Hainan and Malaysia. None of 11 species currently known is similar in external morphology to the Chinese species. Fei et al. (2003) made a morphological study, including detailed osteological and myologic observations, and by comparing data their own and others (e.g., Inger, 1960; McDiarmid, 1971), they erected the new genus *Parapelophryne* to differentiate the species from the other named species.

In this way, the genus was erected purely on the basis of morphological information, considered to have limited utility in estimating phylogenetic relationships (e.g., Hillis and Wiens, 2000; Wiens, 2004). Our results rejected some previously reported relationships such as the clade of *Pelophryne* and *Ansonia* (Matsui et al., 2012), while supporting the clade of *Phrynoidis* and *Pedostibes* as in previous reports. Our results confirmed distinct generic status of *Parapelophryne*, yet indicated its sister generic relationships not to any Southeast Asian genus but to East Asian *Bufo*. This is surprising, because the two genera differ greatly in morphology and ecology (Fei et al., 2003), and occur in regions apart from each other (Fei, 1999).

Members of *Bufo* occur mainly in temperate regions, but some species like *B. cryptotympanicus* Liu and Hu, 1962 and *B. aspinus* (Rao and Yang, 1994) are recorded from southern China and/or northern Vietnam, nearer to Hainan (Fei et al., 2012). However, because these species are not distantly related genetically to *B. japonicus* studied here (Liu et al., 2000; Pyron and Wiens, 2011), examination of relationships between *Parapelophryne* and *Bufo* from southern China is expected to confirm our results. Future studies including South- and East Asian genera not treated here (*Adenomus* Cope, 1861; *Bufo*ides Pillai and Yazdani, 1973; *Bufo*tes Rafinesque, 1814; *Epidalea* Cope, 1864; *Ghatophryne* Biju, Van Bocxlaer, Giri, Loader, and Bossuyt, 2009;



*Pseudobufo* Tschudi, 1838; *Strauchbufo* Fei, Ye, and Jiang, 2012; and *Xanthophryne* Biju, Van Bocxlaer, Giri, Loader, and Bossuyt, 2009) would be more fruitful. Such studies might change our conclusion because their great morphological and ecological differences from *Parapelophryne* may be superficial, thus masking phylogenetic relationships.

#### ACKNOWLEDGMENTS

We thank Hainan Forestry Department for their cooperation and assistance, J.-P. Jiang and H. Ota for supplying valuable information, T. Tanaka-Ueno for laboratory assistance, and two reviewers for improving the manuscript. This research was supported partly by a Grant-in Aid from the Monbusho through the JSPS (Field Research, No. 23405014) to M. Matsui, and Postdoctoral Program of Japan Society for the Promotion of Science (JSPS) to W.-Z. Liu.

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Accepted: 22 June 2015